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(54) Title: SOLUBLE, ACTIVE HEPATITIS C VIRUS PROTEASE

(57) Abstract

Soluble HCV NS3 protease, including the NS3 protease fused to a solubilizing motif. A fusion of the NS3 and NS4 regions under conditions where they are not cleaved by the NS3 protease. Bacterially expressed soluble HCV NS3 protease. Host cells wherein at least 1 % f the cell's total protein is soluble HCV NS3 protease.

<u>SOLUBLE, ACTIVE HEPATITIS C VIRUS PROTEASE</u>

5

<u>BACKGROUND OF THE INVENTION</u>

10   Hepatitis C virus (HCV) is considered to be the major etiological
agent of non-A non-B (NANB) hepatitis, chronic liver disease, and
hepatocellular carcinoma (HCC) around the world. The viral infection
accounts for greater than 90% of transfusion -associated hepatitis in U.S.
and it is the predominant form of hepatitis in adults over 40 years of
15   age. Almost all of the infections result in chronic hepatitis and nearly
20% develop liver cirrhosis.

        The virus particle has not been identified due to the lack of an
efficient *in vitro* replication system and the extremely low amount of
20   HCV particles in infected liver tissues or blood. However, molecular
cloning of the viral genome has been accomplished by isolating the
messenger RNA (mRNA) from the serum of infected chimpanzees then
cloned using recombinant methodologies. [Grakoui A. *et al. J. Virol.* 67:
1385 - 1395 (1993)] It is now known that HCV contains a positive strand
25   RNA genome comprising approximately 9400 nucleotides, whose
organization is similar to that of flaviviruses and pestiviruses . The
genome of HCV, like that of flavi- and pestiviruses, encodes a single
large polyprotein of about 3000 amino acids which undergoes proteolysis
to form mature viral proteins in infected cells.
30
        Cell-free translation of the viral polyprotein and cell culture
expression studies have established that the HCV polyprotein is
processed by cellular and viral proteases to produce the putative
structural and nonstructural (NS) proteins. At least nine mature viral
35   proteins are produced from the polyprotein by specific proteolysis. The
order and nomenclature of the cleavage products are as follows: NH2-C-
E1-E2-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH.(Fig 1). The three
amino terminal putative structural proteins, C (capsid), E1, and E2 (two

envelope glycoproteins), are believed to be cleaved by host signal peptidases of the endoplasmic reticulum(ER) . The host enzyme is also responsible for generating the amino terminus of NS2 . The proteolytic processing of the nonstructural proteins are carried out by the viral

5   proteases: NS2-3 and NS3, contained within the viral polyprotein. The NS2-3 protease catalyzes the cleavage between NS2 and NS3. It is a metalloprotease and requires both NS2 and the protease domain of NS3. The NS3 protease catalyzes the rest of the cleavages in the nonstructural part of the polyprotein. The NS3 protein contains 631 amino acid

10   residues and is comprised of two enzymatic domains: the protease domain contained within amino acid residues 1-181 and a helicase ATPase domain contained within the rest of the protein. It is not known if the 70 kD NS3 protein is cleaved further in infected cells to separate the protease domain from the helicase domain, however, no

15   cleavage has been observed in cell culture expression studies.

The NS3 protease is a member of the serine class of enzymes. It contains His, Asp, and Ser as the catalytic triad, Ser being the active site residue. Mutation of the Ser residue abolishes the cleavages at substrates

20   NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B. The cleavage between NS3 and NS4A is intramolecular, whereas the cleavages at NS 4A/4B, 4B/5A, 5A/5B sites occur in *trans* .

Experiments using transient expression of various forms of HCV

25   NS polyproteins in mammalian cells have established that the NS3 serine protease is necessary but not sufficient for efficient processing of all these cleavages. Like flaviviruses, the HCV NS3 protease also requires a cofactor to catalyze some of these cleavage reactions. In addition to the serine protease NS3, the NS4A protein is absolutely

30   required for the cleavage of the substrate at the 4B/5A site and increases the efficiency of cleavage of the substrate between 5A/5B, and possibly 4A/4B.

Because the HCV NS3 protease cleaves the non-structural HCV

35   proteins which are necessary for the HCV replication, the NS3 protease can be a target for the development of therapeutic agents against the HCV virus. The gene encoding the HCV NS3 protein has been cloned as disclosed in U.S. Patent N . 5,371,017, however, the protein has not

been produced in a soluble active form. If the HCV protease is to be useful as a target in a screen to discover therapeutic agents, the protease must be produced in a soluble active form. Thus, there is a need for a soluble active form of the HCV protease which can be produced in large

5   quantities to be used in high throughput screen to detect inhibitors of the protease and for structural studies.

SUMMARY OF THE INVENTION

10   The present invention fills this need by providing for a soluble, active NS3 protease. In one embodiment of the present invention, the soluble NS3 protease is contained within a fusion protein comprised of a HCV protease fused to a solubilizing motif.

15   The present invention further provides for a soluble fusion protein comprised of the catalytic domain of the NS3 protease, cofactor domain of cofactor NS4A and a solubilizing motif wherein the NS4A cofactor has been mutated so that the NS3 protease and NS4A cofactor are not cleaved by the catalytic activity of the NS3 protease.

20
      The present invention further provides for an HCV NS3 protease having a polypeptide comprising three or more histidine residues fused to the protease. This enables rapid purification of the protease.

25   The present invention provides further for a soluble HCV NS3 protease selected from the group consisting of SEQ ID NO 3, SEQ ID NO 4, SEQ ID NO: 5 SEQ ID NO 7, SEQ ID NO 8, SEQ ID NO 9, SEQ ID NO 10 and SEQ ID NO 27.

30   The present invention further provides for isolated nucleic acids and vectors which encode the HCV proteases of the present invention, host cells transformed or transfected by said nucleic acids or vectors. Also claimed is a method for making a soluble HCV protease comprising culturing the transformed or transfected host cell under

35   conditions in which the nucleic acid or vector is expressed.

      The present invention further provides for a host cell transformed or transfected with a nucleic acid or vector able to express

soluble HCV NS3 protease, wherein the soluble HCV NS3 protease which is expressed is at least 1%, 2% , 3% , 4%, 5% or more of the total protein expressed by the cell.

5

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 schematically depicts the HCV genome.

10   Figure 2 depicts the recombinant synthesis of plasmid pBJ1015.

Figure 3 depicts the recombinant synthesis of plasmid pTS56-9.

Figure 4 depicts the recombinant synthesis of plasmid pJB1006.

15

Figure 5 depicts the recombinant synthesis of plasmid pBJ1022.

Figure 6 depicts the recombinant synthesis of plasmid pNB(-V)182Δ4AHT.

20

Figure 7 depicts the recombinant synthesis of plasmid pT5His/HIV/183.

Figure 8 schematically depicts a high throughput assay for discovering HCV protease inhibitors using surface plasmon resonance technology.

25

DETAILED DESCRIPTION OF THE INVENTION

The teachings of all references cited are incorporated herein in their entirety by reference.

30

The present invention is the production of the HCV NS3 protease in a soluble form. The HCV NS3 protease must be in a soluble form to be used in a screen to detect compounds which inhibit the protease from cleaving it's target substrate. We have discovered that if a peptide

35   containing a solubilizing motif is attached to either the NS3 proteas , preferably to the carboxyl terminus, the NS3 protease becomes readily soluble.

The amino acid sequence of the NS3 protease catalytic domain is shown in SEQ ID NO: 1. Prior to the present invention the NS3 protease was not expressed in a cell in a soluble form in sufficient quantities for extraction and purification. Moreover, soluble HCV NS3

5 protease was not able to be produced in soluble form in bacteria. This is important because bacterial expression is the preferred method of expression of large quantities of HCV protease. Soluble HCV NS3 protease of the present invention can be produced in several ways. A solubilizing motif can be fused to the protein resulting in a soluble

10 protein. A solubilizing motif is any chemical moiety bound to the HCV NS3 protease which results in the NS3 protease becoming soluble in a buffered solution. Examples of such solubilizing motifs are chains of amino acids having polar side chains, preferably positively charged amino acids. The chain of amino acids should be about 4 - 10 amino

15 acid residues in length. The preferred amino acids are arginine and lysine. Another example of a solubilizing motif is an amphipathic moiety. The solubilizing motif can be fused to either the amino terminus or carboxy terminus of the NS3 protease. A sequence which has been successfully fused to the carboxyl terminus to produce soluble

20 NS3 protease is -Arg - Lys - Lys - Lys - Arg - Arg- (SEQ ID NO: 2). This has been fused to the carboxyl end of the NS3 protease to produce the polypeptides of SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 8 and SEQ ID NO: 27. Other examples of soluble HCV NS3 protease having a hydrophilic amino acid residue tail which were made are SEQ ID NO: 9,

25 and SEQ ID NO: 10.

In another embodiment of the present invention, soluble HCV NS3 protease can also be produced which does not have a solubilizing motif as for example the proteases shown in SEQ ID NO: 1 and SEQ ID

30 NO: 7. Preferably the NS3 protease will have a histidine tag fused to its amino acid terminus for use in purifying the protein on a nickel (Ni^{2+}) coated resin. See SEQ ID NO: 5. In this embodiment the protease is produced as insoluble aggregates or as inclusion bodies in bacteria such as in E. coli.

35

The insoluble HCV NS3 protease is first extracted from the bacteria by homogenization or sonication of the bacteria. The aggregates containing the bacteria are then solubilized in a 5 M solution of

guanidine hydrochloride (GuHCl). The NS3 protease is then purified from high molecular weight aggregates by size exclusion chromatography, as for example by applying the solution to a SEPHACRYL S-300 size exclusion gel column. Fractions containing the

5   NS3 protease in 5 M GuCl are pooled and diluted to about 0.1 M GuHCl in a refolding buffer comprised of dithiothreitol and lauryl maltoside. The diluted solution is then applied to a reverse phase chromatography column and pools containing the NS3 protease collected. The pH of the protease fractions is then raised in a stepwise manner to about 7.4 so as

10   to produce properly refolded soluble, active NS3 protease.

It has also been discovered that the HCV NS3 protease is much more effective in cleaving the HCV non-structural proteins, if the co-factor NS4A protein is present (SEQ ID NO: 6). Accordingly, the present

15   invention is also comprised of a fusion of the NS4A cofactor domain protein with the NS3 protease, in particular the fusion of the NS3 protease and the NS4A cofactor wherein the NS4A is mutated such the NS3 protease and the NS4A cofactor is not cleaved by the NS3 protease. Examples of the fused NS3 and NS4A constructs are shown in SEQ ID

20   NOs, 7, 8, 9, 10 and 27.

DNA encoding the NS3 protease of this invention can be prepared by chemical synthesis using the known nucleic acid sequence [Ratner et al., Nucleic Acids Res. 13:5007 (1985)] and

25   standard methods such as the phosphoramidite solid support method of Matteucci et al. [J Am. Chem. Soc. 103:3185 (1981)] or the method of Yoo et al. [J. Biol. Chem. 764:17078 (1989)]. See also Glick, Bernard R. and Pasternak, *Molecular Biotechnology* : pages 55 - 63, (ASM Press, Washington, D.C. 1994). The gene encoding the protease

30   can also be obtained using the plasmid disclosed in Grakoui, A., Wychowski, C., Lin, C., Feinstone, S. M., and Rice, C. M., Expression and Identification of Hepatitis C Virus polyprotein Cleavage Products, *J. Virol* 67;1385-1395 (1993). Also, the nucleic acid encoding HCV protease can be isolated, amplified and cloned (from patients

35   infected with the HCV virus). Furthermore, the HCV genome has been disclosed in PCT WO 89/04669 and are available from the

American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD under ATCC accession no. 40394.

Of course, because of the degeneracy of the genetic code, there are many functionally equivalent nucleic acid sequences that can

5   encode mature human HCV protease as defined herein. Such functionally equivalent sequences, which can readily be prepared using known methods such as chemical synthesis, PCR employing modified primers and site-directed mutagenesis, are within the scope of this invention.

10

Various expression vectors can be used to express DNA encoding HCV NS3 protease. Conventional vectors used for expression of recombinant proteins used for expression of recombinant proteins in prokaryotic or eukaryotic cells may be used. Preferred vectors include

15   the pcD vectors described by Okayama *et al., Mol. Cell. Bio. 3*: 280-289 (1983); and Takebe *et al., Mol. Cell. Biol. 8*: 466-472 (1988). Other SV40-based mammalian expression vectors include those disclosed in Kaufman *et al., Mol. Cell. Biol. 2*: 1304-1319 (1982) and U.S. Patent No. 4,675,285. These SV40-based vectors are particularly useful in COS7

20   monkey cells (ATCC No. CRL 1651), as well as in other mammalian cells such as mouse L cells and CHO cells.

Standard transfection methods can be used to produce eukaryotic cell lines which express large quantities of the polypeptide. Eukaryotic

25   cell lines include mammalian, yeast and insect cell lines. Exemplary mammalian cell lines include COS-7 cells, mouse L cells and Chinese Hamster Ovary (CHO) cells. See Sambrook *et al., supra* and Ausubel *et al., supra.*

30   As used herein, the term "transformed bacteria" means bacteria that have been genetically engineered to produce a mammalian protein. Such genetic engineering usually entails the introduction of an expression vector into a bacterium. The expression vector is capable of autonomous replication and protein expression relative to genes in the

35   bacterial genome. Construction of bacterial expression is well known in the art, provided the nucleotide sequence encoding a desired protein is known or otherwise available. For example, DeBoer in U.S. Pat. No.

4,551,433 discloses promoters for use in bacterial expression vectors; Goeddel *et al.* in U.S. Pat. No. 4,601,980 and Riggs, in U.S. Pat. No. 4,431,739 disclose the production of mammalian proteins by *E. coli* expression systems; and Riggs *supra*, Ferretti *et al. Proc. Natl. Acad.*

5   *Sci.83*:599 (1986), Sproat *et al., Nucleic Acid Research 13*:2959 (1985) and Mullenbach *et al., J. Biol. Chem 261*:719 (1986) disclose how to construct synthetic genes for expression in bacteria. Many bacterial expression vectors are available commercially and through the American Type Culture Collection (ATCC), Rockville, Maryland.

10

Insertion of DNA encoding human HCV protease into a vector is easily accomplished when the termini of both the DNA and the vector comprise the same restriction site. If this is not the case, it may be necessary to modify the termini of the DNA and/or vector by

15   digesting back single-stranded DNA overhangs generated by restriction endonuclease cleavage to produce blunt ends, or to achieve the same result by filling in the single-stranded termini with an appropriate DNA polymerase. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the

20   termini. Such linkers may comprise specific oligonucleotide sequences that define desired restriction sites. The cleaved vector and the DNA fragments may also be modified if required by homopolymeric tailing.

Many *E. coli*-compatible expression vectors can be used to

25   produce soluble HCV NS3 protease of the present invention, including but not limited to vectors containing bacterial or bacteriophage promoters such as the *Tac, Lac, Trp, LacUV5*, 1 P_r and 1 P_L promoters. Preferably, a vector selected will have expression control sequences that permit regulation of the rate of HCV protease

30   expression. Then, HCV protease production can be regulated to avoid overproduction that could prove toxic to the host cells. Most preferred is a vector comprising, from 5' to 3' (upstream to downstream), a *Tac* promoter, a *lac* Iq repressor gene and DNA encoding mature human HCV protease. The vectors chosen for use

35   in this invention may also encode secretory leaders such as the ompA or protein A leader, as long as such leaders are cleaved during

post-translational processing to produce mature HCV protease or if
the leaders ar not cleaved, the leaders do not interfere with the
enzymatic activity of the protease.

5   Fusion peptides will typically be made by either recombinant
nucleic acid methods or by synthetic polypeptide methods. Techniques
for nucleic acid manipulation and expression are described generally,
e.g., in Sambrook, et al. (1989) *Molecular Cloning: A Laboratory Manual*
(2d ed.), vols. 1-3, Cold Spring Harbor Laboratory; and Ausubel, et al.
10   (eds.) (1993) *Current Protocols in Molecular Biology*, Greene and Wiley,
NY. Techniques for synthesis of polypeptides are described, e.g., in
Merrifield (1963) *J. Amer. Chem. Soc. 85*:2149-2156; Merrifield (1986)
Science 232: 341-347; and Stewart et al (1984)., *"Solid Phase Peptide
Synthesis"* (2nd Edition), Pierce Chemical Co., Rockford, IL.; and
15   Atherton, et al. (1989) *Solid Phase Peptide Synthesis: A Practical
Approach*, IRL Press, Oxford; and Grant (1992) *Synthetic Peptides: A
User's Guide*, W.H. Freeman, NY.

      The smaller peptides such as the NS4A cofactor and the substrates
20   5A/5B and 4B/5A can be synthesized by a suitable method such as by
exclusive solid phase synthesis, partial solid phase methods, fragment
condensation or classical solution synthesis. The polypeptides are
preferably prepared by solid phase peptide synthesis as described by
Merrifield, J. Am. Chem. Soc. *85*:2149 (1963). The synthesis is carried out
25   with amino acids that are protected at the alpha-amino terminus.
Trifunctional amino acids with labile side-chains are also protected with
suitable groups to prevent undesired chemical reactions from occurring
during the assembly of the polypeptides. The alpha-amino protecting
group is selectively removed to allow subsequent reaction to take place
30   at the amino-terminus. The conditions for the removal of the alpha-
amino protecting group do not remove the side-chain protecting groups.

      The alpha-amino protecting groups are those known to
be useful in the art of stepwise polypeptide synthesis. Included are
35   acyl type protecting groups (*e.g.*, formyl, trifluoroacetyl, acetyl), aryl
type protecting groups (*e.g.* , biotinyl), aromatic urethane type
protecting groups [*e.g.*, benzyloxycarbonyl (Cbz), substituted
benzyloxycarbonyl and 9-flu renylmethyloxy-carbonyl (Fmoc)],

aliphatic urethane protecting groups [e.g., t-butyloxycarbonyl (tBoc), isopropyloxycarbonyl, cyclohexyloxycarbonyl] and alkyl type protecting groups (e.g., benzyl, triphenylmethyl). The preferred protecting groups are tBoc and Fmoc, thus the peptides are said to be
5   synthesized by tBoc and Fmoc chemistry, respectively.

        The side-chain protecting groups selected must remain intact during coupling and not be removed during the deprotection of the amino-terminus protecting group or during coupling
10   conditions. The side-chain protecting groups must also be removable upon the completion of synthesis, using reaction conditions that will not alter the finished polypeptide. In tBoc chemistry, the side-chain protecting groups for trifunctional amino acids are mostly benzyl based. In Fmoc chemistry, they are mostly
15   tert.-butyl or trityl based.

        In tBoc chemistry, the preferred side-chain protecting groups are tosyl for Arg, cyclohexyl for Asp, 4-methylbenzyl (and acetamidomethyl) for Cys, benzyl for Glu, Ser and Thr,
20   benzyloxymethyl (and dinitrophenyl) for His, 2-Cl-benzyloxycarbonyl for Lys, formyl for Trp and 2-bromobenzyl for Tyr. In Fmoc chemistry, the preferred side-chain protecting groups are 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) or 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg, trityl for
25   Asn, Cys, Gln and His, tert. butyl for Asp, Glu, Ser, Thr and Tyr, tBoc for Lys and Trp.

        For the synthesis of phosphopeptides, either direct or post-assembly incorporation of the phosphate group is used. In the
30   direct incorporation strategy, the phosphate group on Ser, Thr or Tyr may be protected by methyl, benzyl or tert.butyl in Fmoc chemistry or by methyl, benzyl or phenyl in tBoc chemistry. Direct incorporation of phosphotyrosine without phosphate protection can also be used in Fmoc chemistry. In the post-assembly incorporation strategy, the
35   unprotected hydroxyl group of Ser, Thr or Tyr was derivatized on solid phase with di-tert.butyl-, dibenzyl- or dimethyl-N,N'-diisopropylphosphoramidite and then oxidized by tert.butylhydroperoxide.

Solid phase synthesis is usually carried out from the carboxyl-terminus by coupling the alpha-amino protected (side-chain protected) amino acid to a suitable solid support. An ester linkage is

5   formed when the attachment is made to a chloromethyl, chlortrityl or hydroxymethyl resin, and the resulting polypeptide will have a free carboxyl group at the C-terminus. Alternatively, when an amide resin such as benzhydrylamine or p-methylbenzhydrylamine resin (for tBoc chemistry) and Rink amide or PAL resin (for Fmoc

10   chemistry) is used, an amide bond is formed and the resulting polypeptide will have a carboxamide group at the C-terminus. These resins, whether polystyrene- or polyamide-based or polyethyleneglycol-grafted, with or without a handle or linker, with or without the first amino acid attached, are commercially available,

15   and their preparations have been described by Stewart et al (1984)., *"Solid Phase Peptide Synthesis"* (2nd Edition), Pierce Chemical Co., Rockford, IL.; and Bayer & Rapp (1986) *Chem. Pept. Prot.* 3, 3; and Atherton, et al. (1989) *Solid Phase Peptide Synthesis: A Practical Approach,* IRL Press, Oxford.

20

The C-terminal amino acid, protected at the side-chain if necessary and at the alpha-amino group, is attached to a hydroxylmethyl resin using various activating agents including dicyclohexylcarbodiimide (DCC), N,N'-diisopropylcarbodiimide

25   DIPCDI) and carbonyldiimidazole (CDI). It can be attached to chloromethyl or chlorotrityl resin directly in its cesium tetramethylammonium salt form or in the presence of triethylamine (TEA) or diisopropylethylamine (DIEA). First amino acid attachment to an amide resin is the same as amide bond formation

30   during coupling reactions

Following the attachment to the resin support, the alpha-amino protecting group is removed using various reagents depending on the protecting chemistry (*e.g.* , tBoc, Fmoc). The extent

35   of Fmoc removal can be monitored at 300-320 nm or by a conductivity cell. After removal of the alpha-amino protecting group, the remaining protected amino acids are coupled stepwise in the required rder to obtain the desired sequence.

Various activating agents can be used for the coupling reactions including DCC, DIPCDI, 2-chloro-1,3-dimethylimidium hexafluorophosphate (CIP), benzotriazol-1-yl-oxy-tris-

5 (dimethylamino)-phosphonium hexafluorophosphate (BOP) and its pyrrolidine analog (PyBOP), bromo-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBroP), O -(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and its tetrafluoroborate analog (TBTU) or its pyrrolidine analog (HBPyU),

10 O -(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) and its tetrafluoroborate analog (TATU) or pyrrolidine analog (HAPyU). The most common catalytic additives used in coupling reactions include 4-dimethylaminopyridine (DMAP), 3-hydroxy-3,4-dihydro-4-oxo-1,2,3-

15 benzotriazine (HODhbt), N-hydroxybenzotriazole (HOBt) and 1-hydroxy-7-azabenzotriazole (HOAt). Each protected amino acid is used in excess (>2.0 equivalents), and the couplings are usually carried out in N-methylpyrrolidone (NMP) or in DMF, CH$_2$Cl$_2$ or mixtures thereof. The extent of completion of the coupling reaction

20 can be monitored at each stage, e.g., by the ninhydrin reaction as described by Kaiser et al., Anal. Biochem. 34:595 (1970). In cases where incomplete coupling is found, the coupling reaction is extended and repeated and may have chaotropic salts added. The coupling reactions can be performed automatically with

25 commercially available instruments such as ABI model 430A, 431A and 433A peptide synthesizers.

After the entire assembly of the desired polypeptide, the polypeptide-resin is cleaved with a reagent with proper scavengers.

30 The Fmoc peptides are usually cleaved and deprotected by TFA with scavengers (e.g., H$_2$O, ethanedithiol, phenol and thioanisole). The tBoc peptides are usually cleaved and deprotected with liquid HF for 1-2 hours at -5 to 0°C, which cleaves the polypeptide from the resin and removes most of the side-chain protecting groups. Scavengers

35 such as anisole, dimethylsulfide and p-thiocresol are usually used with the liquid HF to prevent cations formed during the cleavage from alkylating and acylating the amino acid residues present in the polypeptide. The formyl group of Trp and dinitrophenyl group of His

need to be removed, respectively, by piperidine and thiophenol in DMF prior to the HF cleavage. The acetamidomethyl group of Cys can be removed by mercury(II) acetate and alternatively by iodine, thallium (III) trifluoroacetate or silver tetrafluoroborate which
5   simultaneously oxidize cysteine to cystine. Other strong acids used for tBoc peptide cleavage and deprotection include trifluoromethanesulfonic acid (TFMSA) and trimethylsilyltrifluoroacetate (TMSOTf).

10   Recombinant DNA methodology can also be used to prepare the polypeptides. The known genetic code, tailored if desired with known preferred codons for more efficient expression in a given host organism, can be used to synthesize oligonucleotides encoding the desired amino acid sequences. The phosphoramidite
15   solid support method of Matteucci et al. , J. Am. Chem. Soc. 103:3185 (1981) or other known methods can be used for such syntheses. The resulting oligonucleotides can be inserted into an appropriate vector and expressed in a compatible host organism.

20   The polypeptides of the invention can be purified using HPLC, gel filtration, ion exchange and partition chromatography, countercurrent distribution or other well known methods. In a preferred embodiment of the present invention the NS3 fusion proteins also contain a histidine tag which facilitates purification using a Ni^+ column as is
25   illustrated below.

One can use the NS3 protease, the NS4 cofactor and the peptide substrates, either 4B/5A or 5A/5B, to develop high throughput assays. These can be used to screen for compounds which inhibit proteolytic
30   activity of the protease. This is carried out by developing techniques for determining whether or not a compound will inhibit the NS3 protease from cleaving the viral substrates. Examples of such synthetic substrates are SEQ ID NOs 16, 17, 18, 19, 20 and 21. If the substrates are not cleaved, the virus cannot replicate. One example of such a high throughput
35   assay is the scintillation proximity assay (SPA). SPA technology involves the use of beads coated with scintillant. Bound to the beads are acceptor molecules such as antibodies, receptors or enzyme substrates which interact with ligands or enzymes in a reversible manner.

For a typical protease assay the substrate peptide is biotinylated at one end and the other end is radiolabelled with low energy emitters such as ^{125}I or [3]H. The labeled substrate is then incubated with the

5   enzyme. Avidin coated SPA beads are then added which bind to the biotin. When the substrate peptide is cleaved by the protease, the radioactive emitter is no longer in proximity to the scintillant bead and no light emission takes place. Inhibitors of the protease will leave the substrate intact and can be identified by the resulting light emission

10   which takes place in their presence.

Another type of protease assay, utilizes the phenomenon of surface plasmon resonance (SPR). A novel, high throughput enzymatic assay utilizing surface plasmon resonance technology has been

15   successfully developed. Using this assay, and a dedicated BIAcore™ instrument, at least 1000 samples per week can be screened for either their enzymatic activity or their inhibitory effects toward the enzymatic activity, in a 96 well plate format. This methodology is readily adaptable to any enzyme-substrate reaction. The advantage of this assay over the

20   SPA assay is that it does not require a radiolabeled peptide substrate.

The following examples are included to illustrate the present invention but not to limit it.

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<div align="center">Examples 1</div>

<div align="center">Production of HCV NS3 Protease</div>

30   A.   Plasmid constructions.

Several plasmids were designed and constructed using standard recombinant DNA techniques (Sambrook,Fritsch & Maniatis) to express the HCV protease in *E. coli* (Fig 2-7). All HCV specific sequences

35   originated from the parental plasmid pBRTM/HCV 1-3011 (Grakoui *et al*.1993). To express the N-terminal 183 amino acid versions f the protease, a stop codon was inserted into the HCV genome using synthetic oligonucleotides (Fig. 3). The plasmids designed to express the

N-terminal 246 amino acid residues were generated by the natural NcoI restriction site at the C-terminus.

i)   Construction of the plasmid pBJ1015 (Figure 2)

The plasmid pBRTM/HCV 1-3011 containing the entire HCV genome (Grakoui A., et al., J. Virol. 67: 1385-1395) was digested with the restriction enzymes Sca I and Hpa I and the 7138 bp (base pair) DNA fragment was isolated and cloned to the Sma I site of pSP72 (Promega) to produce the plasmid,pRJ201. The plasmid pRJ 201 was digested with Msc I and the 2106 bp Msc I fragment was isolated and cloned into the Sma I site of the plasmid pBD7. The resulting plasmid pMBM48 was digested with Kas I and Nco I, and the 734 bp DNA fragment after blunt ending with Klenow polymerase was isolated and cloned into Nco I digested, klenow polymerase treated pTrc HIS B seq expression plasmid (Invitrogen). The ligation regenerated a Nco I site at the 5' end and Nsi I site at the 3' end of HCV sequence. The plasmid pTHB HCV NS3 was then digested with Nco I and Nsi I, and treated with klenow polymerase and T4 DNA polymerase, to produce a blunt ended 738 bp DNA fragment which was isolated and cloned into Asp I cut, klenow polymerase treated expression plasmid pQE30 (HIV). The resulting plasmid pBJ 1015 expresses HCV NS3 (246 amino acids) protease.

(ii)   Construction of the plasmid pTS 56-9 with a stop codon after amino acid 183 (Figure 3)

The plasmid pTHB HCV NS3 was digested with Nco I, treated with klenow polymerase, then digested with Bst Y I; and the DNA fragment containing HCV sequence was isolated and cloned into Sma I and Bgl II digested pSP72. The resulting plasmid pTS 49-27 was then digested with Bgl II and Hpa I and ligated with a double stranded oligonucleotide:

```
GA TCA   CCG GTC TAG ATCT
    T  GGC CAG ATC TAGA
```
(SEQ ID NO 11) to produce pTS 56-9. Thus, a stop codon was placed directly at the end of DNA encoding the protease catalytic domain of the NS3 protein. This enabled the HCV protease to be xpressed independently from the helicase domain of the NS3 protein.

(iii) Construction of the plasmid pJB 1006 Fused with a peptide of positively charged amino acids at the carboxy terminus of NS3 183 (Figure 4).

The plasmid pTS 56-9 was digested with Sph I and Bgl II and the DNA fragment containing HCV sequence was isolated and cloned into a Sph I, Bgl II cut pSP72. The resulting plasmid pJB 1002 digested with Age I and HpaI and ligated to a double stranded oligonucleotide,

```
CCG  GTC  CGG  AAG  AAA  AAG  AGA  CGC  TAG  C
     AG   GCC  TTC  TTT  TTC  TCT  GCG  ATC  G
```

(SEQ ID NO 12), to construct pJB 1006. This fused the hydrophilic, solubilizing motif onto the NS3 protease.

(iv) Construction of the plasmid pBJ 1022 expressing His-NS3(183)-HT in E.coli (Figure 5)

The plasmid pJB 1006 was digested with NgoM I and Nhe I and the 216 bp DNA fragment was isolated and cloned into Ngo M I, Nhe I cut pBJ 1015 to construct plasmid pBJ 1019. The plasmid pBJ 1019 was digested with Nar I and Pvu II, and treated with Klenow polymerase to fill in 5' ends of Nar I fragments. The expression plasmid pQE31 (Invitrogen) was digested with BamH I, blunt ended with Klenow polymerase. The 717 bp Nar I- Pvu II DNA fragment was isolated and ligated to the 2787 bp BamH I/Klenowed -Msc I (Bal I) fragment of the expression plasmid pQE31 (Invitrogen). The recombinant plasmid, pBJ 1022, obtained after transformation into E.coli expresses His NS3(2-183)-HT which does not contain any HIV protease cleavage site sequence. The plasmid also contains a large deletion in the CAT (Chloramphenicol Acetyl Transferase) gene.

(v) Construction of the plasmid pNB(-V)182-Δ4A HT (Figure 6)

The plasmid pMBM 48 was digested with Eag I and Xho I, treated with Klenow polymerase and the 320 bp DNA fragment was isolated and cloned into BamH I cut , blunt ended pSP 72 to construct the plasmid pJB1004. The 320 bp fragment encodes 7 amino acid from carboxy

terminal of NS3(631), all of NS4A, and the amino terminal 46 amino acid of NS4B. The recombinant plasmid pJB1004 was digested with Eag I and Cel 2, blunt ended with Klenow polymerase. The 220 bp DNA fragment was isolated and cloned into the expression plasmid pQE30

5   which was digested with BamH I and blunt ended with Klenow polymerase prior to ligation. The resulting plasmid pJB 1011 was digested with NgoM I and Hind III and ligated to a double stranded oligonucleotide ,

10   CCG GCA   ATT ATA CCT GAC AGG GAG GTT CTC TAC CAG GAA TTC
         GT TAA TAT GGA CTG TCC CTC CAA GAG ATG GTC CTT AAG

     GAT GAG ATG GAA GAG TGC CGG AAG AAA AAG AGA CGC A
     CTA CTC TAC CTT CTC ACG GCC TTC TTT TTC TCT GCG TTC GA
15   (SEQ ID NO 13)

to construct the plasmid pNB 4A HT. The plasmid pNB 4AHT was digested with Msl I and Xba I. The 1218 bp DNA fragment was isolated and cloned into Age I cut, klenow polymerase treated, Xba I cut vector

20   DNA of pBJ 1019. The ligation results in a substitution of the 183rd amino acid residue valine by a glycine residue in NS3, and a deletion of amino terminal three amino acid residues of NS4A at the junction. The recombinant plasmid pNB182Δ4A HT comprising NS3(182aa)-G-NS4A(4-54 amino acid) does not contain NS3/NS4A cleavage site

25   sequence at the junction and is not cleaved by the autocatalytic activity of NS3. Finally the plasmid pNB182Δ4A HT (SEQ ID NO 8) was digested with Stu I and Nhe I, the 803 bp DNA fragment was isolated and cloned into Stu I and Nhe I cut plasmid pBJ 1022. The resulting plasmid pNB(-V)182-Δ4A HT contains a deletion of the HIV sequence from the amino

30   terminus end of the NS3 sequence and in the CAT gene (SEQ ID NO 27).

(vi) Construction of the plasmid pT5 His HIV-NS3 (Figure 7)

         The plasmid pTS56-9 was digested with Bgl II, and treated with

35   Klenow polymerase to fill in 5' ends. The plasmid was then digested with NgoM I and the blunt ended Bgl II/NgoMI fragment containing the NS3 sequence was isolated and ligated to the Sal I, Klenow treated

Ngo MI cut and Sal I klenowed pBJ 1015. Th resulting plasmid is designated pT5His HIV 183.

Example 2

Purification of HCV NS3 Protease having a Solubilizing Motif

Purification of His182HT (SEQ ID NO 4) and His (-V)182Δ4AHT (SEQ ID NO 8)

The recombinant plasmids pBJ1022 and pNB(-V)182Δ4A were used to transform separate cultures of *E. coli* strain M15 [pREP4] (Qiagen), which over-expresses the *lac* repressor, according to methods recommended by the manufacturer. M15 [pREP4] bacteria harboring recombinant plasmids were grown overnight in broth containing 20g/L bactotrypton, 10g/L bacto-yeast extract, 5g/L NaCl and supplemented with 100µg/ml ampicillin and 25µg/ml kanamycin. Cultures were diluted down to O.D.600 of 0.1, then grown at 30°C to O.D.600 of 0.6 to 0.8, after which IPTG was added to a final concentration of 1mM. At post-induction 2 to 3 hours, the cells were harvested by pelleting, and the cell pellets were washed with 100mM Tris, pH 7.5. Cell lysates were prepared as follows: to each ml equivalent of pelleted fermentation broth was added 50µl sonication buffer (50mM sodium phosphate, pH 7.8, 0.3M NaCl) with 1mg/ml lysozyme; cell suspension was placed on ice for 30 min. Suspension was then brought to a final concentration of 0.2% Tween-20, 10mM dithiothreitol (DTT), and sonicated until cell breakage was complete. Insoluble material was pelleted at 12,000 x g in a microcentrifuge for 15 minutes, the soluble portion was removed to a separate tube and the soluble lysate was then brought to a final concentration of 10% glycerol. Soluble lysates from cells expressing the plasmids produce strongly immunoreactive bands of the predicted molecular weight. Soluble lysates prepared for Ni^{2+} column purification were prepared with 10mM β-mercaptoethanol (BME) instead of DTT. Lysates were stored at -80°C.

Purification using Ni^{2+}-Nitrosyl acetic acid (NTA) agarose (QIAGEN)

The proteins were then purified by placing the extracted lysate on
an NTA agarose column. NTA agarose column chromatography was
used because the histidine tag which was fused to the N-terminus of the
proteases readily binds to the nickel column. This produces a powerful
affinity chromatographic technique for rapidly purifying the soluble
protease. The column chromatography was performed in a batch mode.
The Ni^{2+} NTA resin (3ml) was washed twice with 50 ml of Buffer A
(50mM sodium phosphate pH 7.8 containing 10% glycerol, 0.2% Tween-
20, 10mM BME). The lysate obtained from a 250 ml fermentation (12.5
ml) was incubated with the resin for one hour at 4°C. The flow through
was collected by centrifugation. The resin was packed into a 1.0 x 4 cm
column and washed with buffer A until the baseline was reached. The
bound protein was then eluted with a 20 ml gradient of imidazole (0-
0.5M) in buffer A. Eluted fractions were evaluated by SDS-PAGE and
western blot analysis using a rabbit polyclonal antibody to His-HIV 183.
The amount of soluble, active, HCV protease recovered was equal to
about 5% of the total protein expressed by the cells as determined by the
Bradford assay, U.S. Patent No. 4,023,933.

Purification using POROS metal-chelate affinity column

In an alternative method to purify the proteins the lysate containing the
proteins were applied to a POROS metal-chelate affinity column.
Perfusion chromatography was performed on a POROS MC metal
chelate column (4.6 x 50mm, 1.7 ml) precharged with Ni^{2+}. The sample
was applied at 10 ml/min and the column was washed with buffer A.
The column was step eluted with ten column volumes of buffer A
containing 25 mM imidazole. The column was further eluted with a 25
column volume gradient of 25-250 mM imidazole in buffer A. All
eluted fractions were evaluated by SDS-PAGE and western blot analysis
using rabbit polyclonal antibody. The amount of soluble, active, HCV
protease recovered was equal to about 5% of the total protein expressed
by the cells as determined by the *Bradford* assay.

Example 3

Peptide Synthesis of the 5A/5B and 4B/5A Substrates

The peptides 5A/5B and 4B/5A substrates (SEQ ID NOs 16, 18, 19, 20 and
21) were synthesized using Fmoc chemistry on an ABI model 431A
peptide synthesizer. The manufacture recommended FastMoc™
activation strategy (HBTU/HOBt) was used for the synthesis of 4A
activator peptide. A more powerful activator, HATU with or without
the additive HOAt were employed to assemble 5A/5B substrate peptides
on a preloaded Wang resin. The peptides were cleaved off the resin and
deprotected by standard TFA cleavage protocol. The peptides were
purified on reverse phase HPLC and confirmed by mass spectrometric
analysis.

Example 4

HPLC-assay using a synthetic 5A/5B peptide substrate

To test the proteolytic activity of the HCV NS3 protease the
DTEDVVCC SMSYTWTGK (SEQ ID NO 16) and soluble HCV NS3 (SEQ
ID NO 27) were placed together in an assay buffer. The assay buffer was
50mM sodium phosphate pH 7.8, containing 15% glycerol, 10mM DTT,
0.2% Tween20 and 200 mM NaCl). The protease activity of SEQ ID NO
27 cleaved the substrate into two byproduct peptides, namely 5A and 5B.
The substrate and two byproduct peptides were separated on a reversed-
phase HPLC column. (Dynamax, 4.6 x 250 mm) with a pore size of 300Å
and a particle size of 5μm. The column was equilibrated with 0.1%TFA
(Solvent A) at a flow rate of 1 ml per minute. The substrate and the
product peptide standards were applied to the column equilibrated in A.
Elution was performed with a acetonitrile gradient (Solvent B=100%
acetonitrile in A). Two gradients were used for elution (5% to 70%B in
50 minutes followed by 70% to 100%B in 10 minutes).

In another experiment, partially purified SEQ ID NO 27 or vector
control was incubated with 100μM of substrate for 3, 7 and 24 hours at

30°C. The reaction mixture was quenched by the addition of TFA to 0.01% and applied to the reversed-phase HPLC column. The fractions from each run were evaluated by mass spectrometry and sequencing.

5

Example 5

Analysis of NS3 Protease Activity By *In Vitro* Translation Assay

10

To detect HCV NS3 protease activity in *trans*, we have expressed a 40 kD protein containing the NS5A/5B cleavage site in cell-free translation system and used that as the substrate for the enzyme. The substrate protein produces two protein products of apparent molecular weight 12.5 kD (NS 5A') and 27 kD (NS5B') upon cleavage by the HCV

15 NS3 protease.

The plasmid pTS102 encoding the substrate 5A/5B was linearized by digestion with EcoR I and was transcribed using T7 RNA polymerase

20 *in vitro*. The RNA was translated in presence of ^{35}S methionine in rabbit reticulocyte lysates according to the manufacturer's (Promega) protocol to produce HCV specific protein. In a 20 μl total reaction mixture containing 10mM Tris, pH 7.5, 1mM DTT, 0.5mM EDTA, and 10% glycerol was placed 2 to 8 μl of ^{35}S methionine-labeled translated

25 5A/5B substrate. The reaction was started with the addition of 10μl of HCV NS3 protease in solubilization buffer (50mM Na Phosphate, pH 7.8, 0.3M NaCl, 0.2% Tween 20, 10 mM DTT or BME, 10% glycerol), and incubated at 30°C for the specified time. Reactions were stopped by adding an equal volume of 2X Laemmli sample buffer (Enprotech Inc.)

30 and heating at 100°C for 3 minutes. Reaction products were separated by SDS PAGE electrophoresis; gels were fixed, dried and subjected to autoradiography.

The *in vitro* translated substrate was used to assay the HCV NS3

35 proteases expressed by *E. coli* harboring plasmids pBJ1022 and pNB(-V)182Δ4A (SEQ ID NOs 4 and 27). In a two hour assay incubated at 30 C, pBJ1022 crude soluble lysate at 3, 6, and 10μl, was able to cleave 5A/5B substrate in a dose responsive manner, producing the expected

cleaved products: 5A (12.5 kD) and 5B (27 kD) as shown by SDS PAGE analysis. Corresponding vector control lysate did not show any cleavage activity over background. The crude soluble lysate derived from pNB182Δ4A was much more active in this assay. After only 30 minutes

5   incubation, the 5A and 5B cleavage products were detected using as little as 0.125μl cell lysate, with increasing amounts of lysate showing increased cleavage, reaching a maximum at 1μl.

We performed a time course study of the NS3 Protease activity of

10   pNB182Δ4A in an *in vitro* translation assay for further characterization of the activity. At 30°C, in a reaction containing the translated 5A/5B substrate plus pNB182Δ4A soluble lysate at 1μl per 20μl reaction volume, the 5A and 5B cleavage products appeared beginning at 1 minute, and increased with time at 2.5, 5, 10, and 20 minutes.

15

Since we were able to demonstrate HCV NS3 Protease activity using crude cell lysates of pBJ1022 and pNB182Δ4A, we wanted to at least partially purify the expressed proteins in an effort to remove bacterial proteases from these preparations. For this purpose, affinity column

20   chromatography using Ni^{2+} bound ligands was found to be effective, binding the histidine tag at the amino terminal ends of the expressed proteins, and subsequently releasing the bound proteins by imidazole elution. The imidazole-eluted fractions resulting from the purification of pNB182Δ4A on a Ni-NTA column were tested for activity in the *in*

25   *vitro* translation assay. The resultant fractions were all able to cleave the translated 5A/5B Substrate, producing the expected 5A and 5B products. Background bacterial protease activity was not detected in these eluted fractions .

30   As was described above, pBJ1022 was purified by another method of Ni^{2+} chelate chromatography, using POROS Ni^{2+} chelate resin and perfusion chromatography. Imidazole-eluted fractions which were positive for immunoreactivity with antibody to NS3 183 were tested for HCV protease activity by *in vitro* translation assay. In order to optimize

35   detection of activity in this assay for HCV protease, reactions were supplemented with a truncated peptide derived from the NS4A cofactor which has been shown to enhance cleavage at the 5A/5B site by NS3 protease . The cofactor was supplied as a synthetic peptid containing

amino acids 22 to 54 of NS4A (strain HCV-BK) at a final concentration of 1μM. All fractions tested were active in this translation assay.

Example 6

ENHANCEMENT BY 4A PEPTIDES

NS4A is able to enhance the NS3 serine protease activity at NS5A/5B site in mammalian cells that transiently coexpress NS3, NS4A, and the various HCV non-structural polyprotein containing downstream cleavage sites . We have studied this enhancement activity in a well defined cell-free biochemical assay, using the partially purified E.coli-expressed pBJ1022 as a source of NS3 protease, and synthetic peptides containing various truncations of NS4A. In our first experiment we used a crude cell lysate of pBJ1022 as the enzyme and NS4A synthetic peptide truncated 33 mer from amino acid 22 to amino acid 54, the carboxy-terminal in vitro translation cleavage reaction . The C-terminal 33 amino acid peptide of NS4A was able to enhance the activity of the NS3 catalytic domain in a dose dependent manner from 0.01 μM to 1.0 μM peptide, producing the expected products of 5A (12.5kD), and 5B (27kD) from the 40kD translated 5A/5B substrate. Without the 4A peptide a relatively low cleavage activity by the protease alone was observed at the short incubation time of 30 minutes. The 4A peptide itself or with the combination of crude lysate produced from cells harboring the vector plasmid did not cleave the substrate.

To further characterize NS4A enhancement activity additional truncations were made to the NS4A sequence. Truncated peptides were evaluated for their activity in the in vitro translation assay using Ni^{2+} chelate column-purified pBJ1022 (NS3 catalytic domain). We observed that in addition to the C-terminal 33 amino acid peptide, a 18 amino acid peptide containing the NS4A sequence from amino acid 19 through 36 was able to enhance the NS3 mediated cleavage activity. Other peptides, including the N-terminal 21 amino acid, and two shorter truncations from the carboxyl terminal end, a 22mer and a 15mer, were found to have no effect; also a heterologous peptide of 18 amino acid also had no enhancement activity.

Discussion

The experiments described in this report clearly demonstrate that
bacterially expressed HCV protease catalyzes cleavage of i) HCV
polyproteins and ii) synthetic peptide substrates in *trans* biochemical
assay. The processing activity of NS3 catalytic domain is enhanced by
NS4A and its derivatives. The activity of the fusion protein containing
the NS3 catalytic domain and NS4A is much superior to that of the NS3
catalytic domain alone.

Hydrophobicity analysis of the catalytic domain of the NS3
protease reveals that the protein is very hydrophobic and also it contains
seven cysteine residues. To neutralize hydrophobicity and thus to
improve solubility we have added six positively charged amino acid
residues as a solubilizing motif. The addition of a solubilizing motif
appears to improve the solubility without affecting the enzymatic
activity.

We have also shown that the HCV NS4A from Japanese BK
strain has enhanced the HCV-H NS3 mediated cleavage at 5A/5B site.
This suggests that essential elements of recognition may be conserved
among various strains of HCV.

It is clear from above experimental results that attachment of
hydrophilic tail (solubilizing motif/water attracting structures) at the
carboxy terminal end of histidine fused NS3 catalytic domain improved
expression of soluble protein in *E.coli*. In these experiments six residues
of positively charged amino acids are attached at the carboxy terminal
end of the protein. Another example of a solubilizing motif is an
amphipathic helix tail (peptides having charged and hydrophobic
amino acid residues to form both charged and hydrophobic faces) which
is fused to the HCV NS3 protease. Addition of an amphipathic helix at
the carboxy terminus of such fusion proteins will be an alternative way
to achiev improvement of solubility without affecting th enzymatic
activity of the protease.

The hydrophilic tail used in these experiments consists of six amino acids. The sequence and length of the hydrophilic amino acids can be varied to achieve optimal expression of soluble protein. Therefore size of the solubilizing motif and nature of charged residues

5   may effect the expression of soluble NS3 in *E.coli*.

Position of these water attracting structures/motifs at both ends, at one end (amino terminal or carboxy terminal), or insertion within the NS3 catalytic domain and NS3 (catalytic domain)-4A fusion protein,

10   may improve solubility of the protein without affecting the activity.

Based on sequence homology to the members of trypsin superfamily and the protease of other members of the flaviviruses, it is predicted that the amino terminal 181 amino acid of NS3 is the catalytic

15   domain of HCV NS3 protease. Recently it has also been shown that a protein of 169 amino acid containing a 10 amino acid deletion from the amino terminus and 2 amino acid from carboxy terminal of the catalytic domain retains full enzymatic activity. The model we have developed predicts that a protein of 154 amino acids containing a deletion of 26

20   amino acid from amino terminal and a deletion of 2 amino acid from the carboxyl terminus would retain full enzymatic activity for the 5A/5B substrate.

Analysis of the amino acid sequence of the catalytic domain of

25   NS3 protease reveals that the protein contains seven cysteine residues, an odd number, which may cause aggregation. Mutation of one cysteine residue (located on the surface of the protein molecule and not involved in the activity) may improve solubility of the protein without affecting the protease activity.

30

Using the cell free biochemical assay we have demonstrated that the synthetic peptide containing 18 amino acid of HCV NS4A protein is sufficient to enhance the cleavage at NS5A/5B site mediated by the catalytic domain of NS3.

35

Example 7

Refolding of Insoluble HCV NS3 Protease

5

The present example describes a novel process for the refolding of HCV NS3 protease which does not have a solubilizing motif from an E.coli inclusion body pellet. This procedure can be used to generate purified enzyme for activity assays and structural studies.

10

Extraction and Purification of His-HIV 183 from the E.coli inclusion body pellet

15   E. coli cells harboring the plasmid for HisHIV183 was used to transform a culture of E. coli strain M15 [pREP4] (Qiagen), which over-expresses the lac repressor, according to methods recommended by commercial source. M15 [pREP4] bacteria harboring recombinant plasmids were grown overnight in 20-10-5 broth supplemented with 20   100μg/ml ampicillin and 25μg/ml kanamycin. Cultures were diluted to O.D.600 of 0.1, then grown at 37°C to O.D.600 of 0.6 to 0.8, after which IPTG was added to a final concentration of 1mM. At post-induction 2 to 3 hours, the cells were harvested by pelleting, and the cell pellets were washed with 100mM Tris, pH 7.5. were pelleted by centrifugation. The 25   cell pellet was resuspended in 10 ml of 0.1M Tris-HCl, 5mM EDTA, pH 8.0 (Buffer A) for each gm wet weight of pellet. The pellet was homogenized and resuspended using a Dounce homogenizer. The suspension was clarified by centrifugation at 20,000 x g for 30 minutes at 4°C. The pellet was sequentially washed with the following five buffers:

30

1. Buffer A
2. 1.0M sodium chloride (NaCl) in buffer A
3. 1.0% Triton X-100 in buffer A
35   4. Buffer A
5. 1.0 M Guanidine HCl (GuHCl) in buffer A.

The washed pellet was solubilized with 5M GuHCl, 1% beta mercaptoethanol in buffer A (3 ml per gm wet wt. of pellet) using a Dounce homogenizer and centrifuged at 100,000 x g for 30 minutes at 4°C. Purification of denatured HisHIV183 from high

5   molecular weight aggregates was accomplished by size exclusion on a SEPHACRYL S-300 gel filtration column.

In particular, an 8 ml sample of the 5.0M GuHCl *E. coli* extract was applied to a 160 ml Pharmacia S-300 column (1.6 x 100 cm) at a flow

10   rate of 1.0 ml/min. The column buffer was comprised of 5.0 M GuHCl, 0.1 M Tris-HCl, pH 8.0, and 5.0 mM EDTA. The fraction size was 5.0 ml. Appropriate fractions were pooled based on the results of SDS-PAGE, as well as N-terminal sequence analysis of the protein transferred to a Pro-Blot.

15

Detergent-assisted refolding of HCV-protease

The protein was concentrated by ultrafiltration using a 43 mm Amicon YM10 membrane to 1.0 mg per ml in 5M GuHCl, 0.1M Tris-HCl

20   pH 8.0, 1.0 mM EDTA, 1.0% beta-mercaptoethanol. It .was then diluted 50-fold to 0.1M GuHCl in refolding buffer (100 mM sodium phosphate pH 8.0, 10mM DTT, 0.1% lauryl maltoside) and the mixture was incubated on ice for at least one hour. A 25 ml sample containing 500 μg of the protein in the refolding buffer was applied to a Pro-RPC HR 3/5

25   reversed phase chromatography column. The applied sample contained 500 μg protein in 25 ml of refolding buffer. To the column was then applied a solution B comprised of 99.9% H_2O + 0.1% trifluoroacetic acid (TFA). A 10 ml volume of solution C [10% H_2O, 90% acetonitrile (AcN) + 0.1% TFA] was applied to the column at a 0 - 60% gradient into

30   solution B at a flow rate of 0.5ml/min. and a fraction size of 0.5ml. The fractions were monitored at A214; 2.0 absorbance units full scale (AUFS).

Fractions containing the protein (corresponding to peak 1) were pooled for renaturation by stepwise dialysis. The fractions were first

35   dialysed in 0.1% TFA in 25% glycerol overnight at 4°C ; then dialyzed in 0.01% TFA in 25% glycerol overnight at 4°C; then dialyzed in 0.001% TFA in 25% glycerol for 3.0 hours; then dialyzed for 3 hours at 4°C in 50 mM $NaPO_4$, pH 6.0, 10 mM dithiotreitol (DTT) in 25% glycerol. The

protein was then dialyzed for 3.0 hours at 4 C in 50 mM NaPO$_4$, pH 7.0, 0.15 M NaCl, 10 mM DTT in 25% glycerol; and then finally dialyzed in 50 mM NaPO$_4$, pH 7.8, 0.3 M NaCl, 10 mM DTT, 0.2% Tween 20 in 25% glycerol. This resulted in purified, refolded, soluble, active HCV NS3
5   protease.

Far UV circular dichroism (CD) analysis of the protein was used to monitor the refolding from an acid denatured state to a folded state at neutral pH. The protein recovery was monitored by a UV scan and SDS-
10   PAGE analysis.

Results:

Detergent-assisted Refolding of His-HIV183
15

HisHIV183 was quantitatively extracted from an *E. coli* inclusion body pellet. SDS-PAGE analysis at the various stages of extraction shows that sequential washes are essential to remove significant amounts of the contaminating proteins. HisHIV183 was extracted from the washed
20   inclusion body pellet in the presence of 5M GuHCl. The 5M GuHCl extract was applied to a SEPHACRYL S-300 column and the appropriate fractions were pooled based on SDS-PAGE analysis. The amino acid sequence of the first ten residues was verified.

25   Refolding was performed at very low concentrations of protein, in the presence of DTT, lauryl maltoside and glycerol at 4°C. The diluted protein was concentrated on a Pro-RPC reversed phase column. Two peaks were obtained based on the UV and protein profile. Only Peak 1 has yielded soluble protein after stepwise dialysis. Far UV CD spectral
30   analysis was used to monitor refolding from a denatured state at acid pH to a folded state at neutral pH. At pH 7.4, the protein was found to exhibit significant amounts of secondary structure that is consistent with that of beta sheet protein. At low pH, the CD spectrum showed that it is fully random coil, having a minimal molar ellipticity at 200nm. The
35   ratio of this minimum at 200nm to that of the shoulder at 220 nm is approximately 4:1. This ratio decreased when the secondary structure formation occurred at neutral pH.

A UV scan at each step of dialysis showed that the protein recovery was >90% up to pH 7.4 and that there was no light scattering effect due to protein aggregates. SDS-PAGE analysis also indicated that there was no loss of protein up to pH 7.0 during refolding. Precipitation

5   of protein occurred at the last step of dialysis, and the soluble protein was clarified by centrifugation. The overall protein recovery was about 0.10%. The refolded protein was found to be active in a trans-cleavage assay using the *in vitro*-translated 5A/5B substrate in the presence of 4A peptide.

10

Example 8

Analysis of Refolded NS3 Protease Activity by *In Vitro* Translation Assay

15

To detect HCV NS3 protease activity in *trans*, we have expressed a 40 kD protein containing the NS5A/5B cleavage site in cell-free translation system and used that as the substrate for the enzyme. The substrate protein produces two protein products of apparent molecular

20   weight 12.5 kD (NS 5A') and 27 kD (NS5B') upon cleavage by the HCV NS3 protease.

The plasmid pTS102 encoding the substrate 5A/5B was linearized by digestion with EcoR I and was transcribed using T7 RNA polymerase

25   *in vitro*. The RNA was translated in presence of ^{35}S methionine in rabbit reticulocyte lysates according to the manufacturer's (Promega) protocol to produce HCV specific protein. In a 20 µl total reaction mixture containing 10mM Tris, pH 7.5, 1mM DTT, 0.5mM EDTA, and 10% glycerol was placed 2 to 8 µl of ^{35}S methionine-labeled translated

30   5A/5B substrate. The reaction was started with the addition of 10µl of HCV NS3 protease (SEQ ID NO: 5) with an approximately equimolar amount (2 µM) of the carboxyterminal 33 mer cofactor NS4A (SEQ ID NO: 29) in solubilization buffer (50mM Na Phosphate, pH 7.8, 0.3M NaCl, 0.2% Tween 20, 10 mM DTT or BME, 10% glycerol), and incubated

35   at 30°C for about one hour. Reactions were stopped by adding an equal volume of 2X Laemmli sample buffer (Enprotech Inc.) and heating at 100 C for 3 minutes. Reaction products were separated by SDS PAGE electrophoresis; gels were fixed, dried and subjected to autoradiography.

The assay was able to cleave 5A/5B substrate in a dose responsive manner, producing the expected cleaved products: 5A (12.5 kD) and 5B (27 kD) as shown by SDS PAGE analysis. The production of cleaved 5A

5   and 5B polypeptides from the 5A/5B substrate is proof that soluble, active, refolded HCV protease was indeed produced by the process of example 7.

10   Example 9

Surface Plasmon Resonance Assay

The present example illustrates a method for determining if a
15   compound can be useful as an HCV protease inhibitor using the surfac plasmon resonance assay. Figures 8A and 8B illustrate the technique.

BIAcore™ is a processing unit for Biospecific Interaction Analysis. The processing unit integrates an optical detection system
20   with an autosampler and a microfluidic system. BIAcore™ uses the optical phenomena, surface plasmon resonance to monitor interaction between biomolecules. SPR is a resonance phenomenon between incoming photons and electrons on the surface of thin metal film. Resonance occurs at a sharply defined angle of incident light. At this
25   angle, called the resonance angle, energy is transferred to the electrons in the metal film, resulting in a decreased intensity of the reflected light. SPR response depends on a change in refractive index in the close vicinity of the sensor chip surface, and is proportional to the mass of analyte bound to the surface. BIAcore continuously measure the
30   resonance angle by a relative scale of resonance units (RU) and displays it as an SPR signal in a sensorgram, where RU are plotted as a function of time.

In addition, BIAcore™ uses continuous flow technology. One
35   interactant is immobilized irreversibly on the sensor chip, comprising a non-crosslinked carboxymethylated dextran providing a hydrophilic nvironment for bimolecular interaction. Solution containing the other interactant flow continuously over the sensor chip surface. As

molecules from the solution bind to the immobilized ligand, the resonance angle changes resulting in a signal registered by the instrument.

5   In this methodology, the enzymatic reactions are carried out outside of the BIAcore, i.e. in reaction tubes or 96-well tissue culture plates, as it is conventionally done for any of the currently available high throughput assays. The SPR is only used as a detection means for determination of the amount of an intact substrate remaining in a

10   solution with and without the enzyme after the reaction is quenched.

In order to measure the amount of the intact substrate prior to the addition of enzyme, a means of capturing the substrate onto the sensor chip had to be established. In addition, to satisfy the requirement for a

15   high throughput assay on the BIAcore, the substrate needed to be removed from the surface subsequent to completion of analysis. This is required since the same surface will be used for the subsequent reactions. To accomplish these two requirements, a phosphotyrosine is synthetically attached to one end of the substrate. The phosphotyrosine

20   was chosen due to the commercial availability of an anti-phosphotyrosine monoclonal antibody. The antibody is covalently attached to the sensor chip by standard amine coupling chemistry. The anti-phosphotyrosine antibody, bound permanently to the chip is used to capture the phosphotyrosine-containing substrate in a reversible

25   manner. The antibody-phosphotyrosine interaction is ultimately used to capture and release the peptide substrate when desired by regeneration of the surface with various reagents i.e. 2 M $MgCl_2$.

Introduction of the intact peptide onto the antibody surface

30   results in a larger mass which is detected by the instrument. To follow the extent of peptide cleavage, a mixture of peptide substrate and enzyme is incubated for the desired time and then quenched. Introduction of this mixture containing the cleaved peptide and the intact peptide to a regenerated antibody surface results in a lower mass

35   value than that detected for a sample containing only intact peptide. The difference in the two values is then used to calculate the exact amount of intact peptide remaining after cleavage by the enzyme.

Although the reduction in mass can be dir ctly followed with many large substrates, due to the small mass of a typical synthetic peptide substrate (10-20 amino acids, 1-3 Daltons), the mass difference, and thus the signal difference between the intact and cleaved peptide is
5   very small within the signal to noise ratio of the instrument. To circumvent this low sensitivity, we attached a biotin at the N-terminus of the peptide. By addition and thus tagging of peptide with streptavidin prior to injection of tagged peptide onto the antibody surface of the chip, the signal due to the presence of streptavidin will be higher. Using this
10 approach, a cleaved peptide lacking the N-terminal half, tagged with streptavidin will result in a much lower signal.

The HCV protease 5A-5B peptide substrate, DTEDVVACSMSYTWTGK (SEQ ID NO 18) was synthesized with an
15 additional phosphotyrosine at the C-terminus and biotin at the N-terminus.   The biotin was then tagged with streptavidin. An anti-phosphotyrosine monoclonal antibody, 4G10 (Upstate Biotechnology Inc., Lake Placid, New York) was coupled to the sensor chip. In the absence of HCV protease, the intact, streptavidin-tagged biotinylated
20 phosphotyrosine peptide results in a large signal (large mass unit/large signal) through its interaction with the anti-phosphotyrosine monoclonal antibody (Mab).

The protease-catalyzed hydrolysis of the phosphotyrosine-
25 biotinylated peptide was carried out in a 96 well plate. The reaction was stopped with an equal volume of mercuribenzoate. The cleaved peptide which lacks the tagged streptavidin (less mass) results in the loss of response units (lower signal).

30 Using this method, numerous compounds can be tested for their inhibitory activity since the antibody surface can be regenerated repetitively with 2 M $MgCl_2$.

Procedure for Coupling Anti-phosphotyrosine Mab to the Sensor Chip
35

The anti-phosphotyrosine Mab is coupled to the carboxymethylated dextran surface of a sensor chip in the following manner. The flow rate used throughout the coupling procedure is 5

μl/min. The surface is first activated with a 35 μl injection of NHS/EDC (N-hydroxysuccinimide/N-dimethyllaminopropyl-N'-ethylcarbodiimide-HCl). This is followed by a 40 ml injection of Mab 4G10 at 50 μg/ml in 10 mM sodium acetate buffer, pH=4.0. Any

5   remaining activated esters are then blocked by the injection of 35 μl of 1 M ethanolamine. These conditions result in the immobilization of approximately 7,500 response units (420 μM) of antibody.

Binding of Peptide and Regeneration of Mab 4G10 Surface

10

The flow rate used throughout the BIAcore analysis run is 5 μl/min. A 4 μl injection containing streptavidin-tagged peptide (peptide concentration at 2μM, streptavidin binding sites concentration at 9μM) is carried out. The amount of streptavidin-tagged peptide

15   bound to the antibody surface (in response units) is measured 30 seconds after the injection is complete.

Regeneration of sensor chip surface

20   Regeneration of the Mab 4G10 surface is achieved using a 4 μl pulse of 2 M $MgCl_2$ after each peptide injection. Surfaces regenerated up to 500 times still showed 100% binding of tagged peptide.

Determination of the Optimal Concentration of Peptide and
25   Streptavidin

To determine the optimal peptide concentration, a standard curve was generated using various amounts of peptide (0-10 μM) in the presence of excess streptavidin. A value in the linear range, 2 μM, was
30   chosen for standard assay conditions.

The amount of streptavidin required to completely tag the peptide was determined using a peptide concentration of 2.5 μM and titrating the amount of streptavidin (μM of binding sites). All the
35   peptides were shown to be completely tagged when streptavidin concentrations greater than 3 μM (approximately equimolar to the peptide concentration) were used. A streptavidin concentration of 9 μM (a 4.5 fold excess) was chosen for standard assay conditi ns.

Application of Described Methodology to HCV Protease

5

The HCV protease 5A/5B peptide substrate, DTEDVVACSMSYTWTGK (SEQ ID NO 18), with phophotyrosine at the C-terminal and biotin at the N-terminal is synthesized. Anti-phosphotyrosine monoclonal antibody, 4G10 was coupled to the sensor

10 chip.

In the absence of HCV protease, the intact streptavidin-tagged biotinylated phosphotyrosine peptide results in a large signal (large mass unit/large response units) through its interaction with the anti-

15 phosphotyrosine monoclonal antibody.

The protease-catalyzed hydrolysis of the phosphotyrosine-biotinylated peptide was carried out in a 96 well plate. The reaction was stopped with an equal volume of the quenching buffer containing

20 mercuribenzoate. Streptavidin was added to tag the peptide which binds to the biotin. The cleaved peptide which lacks the tagged streptavidin (less mass) results in the loss of response units.

Using this assay, numerous compounds can be tested for their

25 inhibitory activity since the antibody surface can be regenerated repetitively with 2 M MgCl$_2$.

The peptide cleavage activity by HCV protease can be monitored in a time dependent manner using the BIAcore-based methodology.

30 Using the concentrated enzyme and the BIAcore substrate, Biotin-DTEDVVAC SMSYTWTGK-pY (SEQ ID NO 17), 50% substrate cleavage is achieved within 1 hour using the BIAcore-based HCV assay. Based on the amount of enzyme, His-NS3(183)Δ4AHT needed to reach a 50% cleavage within 2 hours, a time scale desired for a development of a

35 high throughput assay, we estimate that 1 liter of fermentation of the His-NS3(183)Δ4AHT construct results in enough protease to run at least 100 reactions on the BIAcore.

Standard Operating Procedure for BIAcore-based HCV Assay

Reactions are prepared in a 96-well tissue culture plate using the Reaction Buffer (50 mM HEPES, pH 7.4, 20 % glycerol, 150 mM NaCl, 1mM EDTA, 0.1% Tween-20,1 mM DTT) as diluent. The final reaction volume is 100 µl. Sample with the peptide alone (Biotin-DTEDVVAC SMSYTWTGKpY) is prepared by addition of 10 µl of peptide stock at 100 µM (prepared in the reaction buffer) to 90 µl of reaction buffer, so that the final concentration of peptide is 10 µM. Samples comprised of peptide and the enzyme are prepared by addition of 10 µl of peptide stock at 100 µM and 10 µl of partially purified His-NS3 (183)-Δ4A-HT stock at 1.7 mg/ml (both prepared in the reaction buffer) to 80 µl of reaction buffer, so that the final concentration of peptide and the enzyme is 10 and 0.1 µM respectively. The reaction is held at 30°C for the specified time and then quenched. Quenching is achieved by transferring a 20-µl aliquot of the reaction mixture to a new tissue culture plate containing an equal volume of PMB Quenching Buffer (50 mM HEPES, pH 7.8, 150 mM NaCl, 5 mM P-Hydroxymercuribenzoic Acid, and 13 mM EDTA).

To prepare the quenched reaction mixture for injection onto the sensor surface, 30 µl PMB BIAcore Buffer (50 mM HEPES, pH 7.4, 1 M NaCl) and 30 µl of streptavidin at 0.5 mg/ml in water is added to the 40 µl of the quenched reaction mixture to a final volume of 100 µl. In this step, all the peptides are tagged with streptavidin prior to the injection of samples. Finally, 4 µl of this sample is injected over the antiphosphotyrosin surface for determination of the intact versus cleaved peptide. The final concentration of peptide and the streptavidin in the BIAcore sample is 2 and 9 µM respectively.

Experimental Conditions:

5	Substrate: ID	Biotin-DTEDVVAC SMSYTWTGK-pY (SEQ NO 19) in Reaction buffer without DTT
	Concentration:	170 μM (Crude peptide, based on weight)
10	Enzyme:	10 μl of concentrated His-NS3 (183)-Δ4A-HT at 1.7 mg/ml
	Reaction volume:	100 μl
15	Reaction buffer:	50 mM HEPES, pH 7.8 20 % glycerol 150 mM NaCl 1mM EDTA 1mM DTT
20		0.1% Tween-20
	Temp:	30° C
25	Quench with:	p-hydroxymercuribenzoate

30

SEQUENCE LISTING

5   (1) GENERAL INFORMATION:

    (i) APPLICANT: Schering Corporation

10   (ii) TITLE OF INVENTION: Hepatitis C Protease Having a Hydrophilic Motif

    (iii) NUMBER OF SEQUENCES: 27

15   (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: Schering Corp.
        (B) STREET: 2000 Galloping Hill Road
        (C) CITY: Kenilworth
        (D) STATE: New Jersey
20   (E) COUNTRY: USA
        (F) ZIP: 07033-0530

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
25   (B) COMPUTER: Apple Macintosh
        (C) OPERATING SYSTEM: Macintosh 7.1
        (D) SOFTWARE: Microsoft Word 5.1a

    (vi) CURRENT APPLICATION DATA:
30   (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (vii) PRIOR APPLICATION DATA:
35   (A) APPLICATION NUMBER: 08/440,409
        (B) FILING DATE: 12 MAY 1995

    (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Lunn, Paul G.
(B) REGISTRATION NUMBER: 32,743
(C) REFERENCE/DOCKET NUMBER: JB0494

5   (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 908-298-5061
(B) TELEFAX: 908-298-5388

(2) INFORMATION FOR SEQ ID NO:1:

10

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 549 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
15   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
20   (A) NAME/KEY: HCV NS3 Protease

```
GCG CCC ATC ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG  45
Ala Pro Ile Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly
 1            5                  10                  15

TGT ATA ATC ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG  90
Cys Ile Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu
             20                  25                  30

GGT GAG GTC CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA 135
Gly Glu Val Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala
             35                  40                  45

ACG TGC ATC AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA 180
Thr Cys Il  Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly
             50                  55                  60
```

```
      ACG AGG ACC ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT 225
      Thr Arg Thr Ile Ala Ser Pro Lys Gly Pro Val Il  Gln Met Tyr
                      65              70              75
  5

      ACC AAT GTG GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT 270
      Thr Asn Val Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly
                      80              85              90

 10   TCC CGC TCA TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC 315
      Ser Arg Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr
                      95              100             105

      CTG GTT ACG AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT 360
 15   Leu Val Thr Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly
                      110             115             120

      GAT AGC AGG GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA 405
      Asp Ser Arg Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu
 20                   125             130             135

      AAA GGC TCC TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC 450
      Lys Gly Ser Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala
                      140             145             150
 25

      GTG GGC CTA TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG 495
      Val Gly Leu Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys
                      155             160             165

 30   GCG GTG GAC TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA 540
      Ala Val Asp Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg
                      170             175             180


      TCC CCG GTG
 35   S r Pro Val
```

(2) INFORMATION FOR SEQ ID NO:2:

5   (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 6 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

10

    (ii) MOLECULE TYPE: peptide

Arg Lys Lys Lys Arg Arg

15   (2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 567 base pairs
        (B) TYPE: nucleic acid
20   (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

25   (ix) FEATURE:
        (A) NAME/KEY:

GCG CCC ATC ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG 45
Ala Pro Ile Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly
30 1   5   10   15

TGT ATA ATC ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG 90
Cys Ile Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu
            20   25   30

35

GGT GAG GTC CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA 135
Gly Glu Val Gln Il Val S r Thr Ala Thr Gln Thr Phe Leu Ala
            35   40   45

```
      ACG TGC ATC AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA 180
      Thr Cys Ile Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly
                      50              55              60

5
      ACG AGG ACC ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT 225
      Thr Arg Thr Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr
                      65              70              75

10    ACC AAT GTG GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT 270
      Thr Asn Val Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly
                      80              85              90


      TCC CGC TCA TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC 315
15    Ser Arg Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr
                      95              100             105


      CTG GTT ACG AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT 360
      Leu Val Thr Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly
20                    110             115             120


      GAT AGC AGG GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA 405
      Asp Ser Arg Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu
                      125             130             135
25
      AAA GGC TCC TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC 450
      Lys Gly Ser Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala
                      140             145             150


30    GTG GGC CTA TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG 495
      Val Gly Leu Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys
                      155             160             165


      GCG GTG GAC TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA 540
35    Ala Val Asp Phe Ile Pro Val Glu Asn Leu Glu Thr Thr M t Arg
                      170             175             180
```

```
TCC CCG GTG AGA AAG AAG AAG AGA AGA
S   r   Pro Val Arg Lys Lys Lys Arg Arg
```

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 603 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (ix) FEATURE:
        (A) NAME/KEY: pBJ1022(His/NS3 (182)/H.T.


```
ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC ACG GAT CCG CCC ATC  45
Met Arg Gly Ser His His His His His His Thr Asp Pro Pro Ile
1               5                   10                  15


ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG TGT ATA ATC  90
Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly Cys Ile Ile
                20                  25                  30


ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG GGT GAG GTC 135
Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu Gly Glu Val
                35                  40                  45


CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA ACG TGC ATC 180
Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala Thr Cys Ile
                50                  55                  60


AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA ACG AGG ACC 225
Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly Thr Arg Thr
                65                  70                  75
```

```
    ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT ACC AAT GTG 270
    Il  Ala S r Pro Lys Gly Pro Val Ile Gln Met Tyr Thr Asn Val
                     80                  85                  90


 5  GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT TCC CGC TCA 315
    Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly Ser Arg Ser
                     95                 100                 105


    TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC CTG GTT ACG 360
10  Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr
                    110                 115                 120


    AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT GAT AGC AGG 405
    Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser Arg
15                  125                 130                 135


    GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA AAA GGC TCC 450
    Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly Ser
                    140                 145                 150

20
    TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC GTG GGC CTA 495
    Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala Val Gly Leu
                    155                 160                 165


25  TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG GCG GTG GAC 540
    Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys Ala Val Asp
                    170                 175                 180


    TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA TCC CCG GTG 585
30  Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg Ser Pro Val
                    185                 190                 195


    AGA AAG AAG AAG AGA AGA
    Arg Lys Lys Lys Arg Arg
35
```

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 630 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (ix) FEATURE:
        (A) NAME/KEY: pT5His/HIV/183 No solubilizing motif

```
ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC GGA TCC CAT AAG GCA   45
Met Arg Gly Ser His His His His His His Gly Ser His Lys Ala
1               5                   10                  15


AGA GTT TTG GCT GAA GCA ATG AGC CAT GGT ACC ATG GCG CCC ATC   90
Arg Val Leu Ala Glu Ala Met Ser His Gly Thr Met Ala Pro Ile
                    20                  25                  30


ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG TGT ATA ATC  135
Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly Cys Ile Ile
                    35                  40                  45


ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG GGT GAG GTC  180
Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu Gly Glu Val
                    50                  55                  60


CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA ACG TGC ATC  225
Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala Thr Cys Ile
                    65                  70                  75


AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA ACG AGG ACC  270
Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly Thr Arg Thr
                    80                  85                  90
```

ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT ACC AAT GTG   315
Ile Ala S r Pro Lys Gly Pro Val Ile Gln Met Tyr Thr Asn Val
                95   100   105

5   GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT TCC CGC TCA   360
    Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly Ser Arg Ser
                    110   115   120

    TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC CTG GTT ACG   405
10 Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr
                    125   130   135

    AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT GAT AGC AGG   450
    Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser Arg
15   140   145   150

    GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA AAA GGC TCC   495
    Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly Ser
                    155   160   165
20
    TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC GTG GGC CTA   540
    Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala Val Gly Leu
                    170   175   180

25 TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG GCG GTG GAC   585
    Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys Ala Val Asp
                    185   190   195

    TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA TCC CCG GTG   630
30 Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg Ser Pro Val
                    200   205   210

35

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 162 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (ix) FEATURE:
        (A) NAME/KEY: NS4A

```
AGC ACC TGG GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG   45
Ser Thr Trp Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala
 1               5                  10                  15


TAT TGC CTG TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC   90
Tyr Cys Leu Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val
                20                  25                  30


TTG TCC GGG AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC  135
Leu Ser Gly Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr
                35                  40                  45


CAG GAG TTC GAT GAG ATG GAA GAG TGC  162
Gln Glu Phe Asp Glu Met Glu Glu Cys
                50
```

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 702 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
      (A) NAME/KEY: NS3 +NS4A

5

```
GCG CCC ATC ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG   45
Ala Pro Ile Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly
 1               5                   10                  15
```

10
```
TGT ATA ATC ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG   90
Cys Ile Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu
                20                  25                  30
```

```
GGT GAG GTC CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA  135
```
15
```
Gly Glu Val Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala
                35                  40                  45
```

```
ACG TGC ATC AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA  180
Thr Cys Ile Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly
                50                  55                  60
```
20

```
ACG AGG ACC ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT  225
Thr Arg Thr Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr
                65                  70                  75
```
25
```
ACC AAT GTG GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT  270
Thr Asn Val Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly
                80                  85                  90
```

30
```
TCC CGC TCA TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC  315
Ser Arg Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr
                95                  100                 105
```

```
CTG GTT ACG AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT  360
```
35
```
Leu Val Thr Arg His Ala Asp Val Il  Pro Val Arg Arg Arg Gly
                110                 115                 120
```

```
GAT AGC AGG GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA 405
Asp S r Arg Gly S r Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu
              125             130             135

AAA GGC TCC TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC 450
Lys Gly Ser Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala
              140             145             150

GTG GGC CTA TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG 495
Val Gly Leu Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys
              155             160             165

GCG GTG GAC TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA 540
Ala Val Asp Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg
              170             175             180

TCC CCG GGG GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG 585
Ser Pro Gly Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala
              185             190             195

TAT TGC CTG TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC 630
Tyr Cys Leu Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val
              200             205             210

TTG TCC GGG AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC 675
Leu Ser Gly Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr
              215             220             225

CAG GAG TTC GAT GAG ATG GAA GAG TGC   702
Gln Glu Phe Asp Glu Met Glu Glu Cys
              230
```

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(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 855 base pairs
5   (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA
10

    (ix) FEATURE:
      (A) NAME/KEY: pNB182Δ4AHT

15 ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC GGA TCC CAT AAG GCA 45
    Met Arg Gly Ser His His His His His His Gly Ser His Lys Ala
    1   5   10   15

    AGA GTT TTG GCT GAA GCA ATG AGC CAT GGT ACC ATG GCG CCC ATC 90
20 Arg Val Leu Ala Glu Ala Met Ser His Gly Thr Met Ala Pro Ile
              20   25   30

    ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG TGT ATA ATC 135
    Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly Cys Ile Ile
25   35   40   45

    ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG GGT GAG GTC 180
    Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu Gly Glu Val
              50   55   60
30

    CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA ACG TGC ATC 225
    Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala Thr Cys Ile
              65   70   75

35 AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA ACG AGG ACC 270
    Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly Thr Arg Thr
              80   85   90

```
    ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT ACC AAT GTG   315
    Ile Ala S r Pro Lys Gly Pro Val Ile Gln Met Tyr Thr Asn Val
                    95              100             105
 5

    GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT TCC CGC TCA   360
    Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly Ser Arg Ser
                    110             115             120

10  TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC CTG GTT ACG   405
    Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr
                    125             130             135

    AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT GAT AGC AGG   450
15  Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser Arg
                    140             145             150

    GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA AAA GGC TCC   495
    Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly Ser
20                  155             160             165


    TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC GTG GGC CTA   540
    Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala Val Gly Leu
25                  170             175             180

    TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG GCG GTG GAC   585
    Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys Ala Val Asp
                    185             190             195
30
    TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA TCC CCG GGG   630
    Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg Ser Pro Gly
                    200             205             210


35  GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG TAT TGC CTG   720
    Val L u Val Gly Gly Val Leu Ala Ala Leu Ala Ala Tyr Cys Leu
                    215             220             225
```

```
    TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC TTG TCC GGG 765
    Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val Leu Ser Gly
                    230             235             240


5   AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC CAG GAG TTC 810
    Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr Gln Glu Phe
                    245             250             255


    GAT GAG ATG GAA GAG TGC CGG AAG AAA AAG AGA CGC AAG CTT AAT 855
10  Asp Glu Met Glu Glu Cys Arg Lys Lys Lys Arg Arg Lys Leu Asn
                    260
```

15

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 711 base pairs
20   (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA
25

    (ix) FEATURE:
      (A) NAME/KEY:

```
    GCG CCC ATC ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG 45
    Ala Pro Ile Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly
30  1           5               10              15


    TGT ATA ATC ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG 90
    Cys Ile Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu
                    20              25              30
35

    GGT GAG GTC CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA 135
    Gly Glu Val Gln Ile Val Ser Thr Ala Thr Gln Thr Ph  Leu Ala
                    35              40              45
```

```
ACG TGC ATC AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA 180
Thr Cys Ile Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly
             50              55              60
```

5

```
ACG AGG ACC ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT 225
Thr Arg Thr Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr
             65              70              75
```

10
```
ACC AAT GTG GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT 270
Thr Asn Val Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly
             80              85              90
```

```
TCC CGC TCA TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC 315
```
15
```
Ser Arg Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr
             95              100             105
```

```
CTG GTT ACG AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT 360
Leu Val Thr Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly
```
20
```
             110             115             120
```

```
GAT AGC AGG GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA 405
Asp Ser Arg Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu
             125             130             135
```

25
```
AAA GGC TCC TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC 450
Lys Gly Ser Ser Gly Gly Pro Leu Leu Cys Pro Ala Gly His Ala
             140             145             150
```

30
```
GTG GGC CTA TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG 495
Val Gly Leu Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys
             155             160             165
```

```
GCG GTG GAC TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA 540
```
35
```
Ala Val Asp Phe Il  Pro Val Glu Asn Leu Glu Thr Thr M t Arg
             170             175             180
```

```
    TCC CCG GGG GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG 585
    Ser Pro Gly Val L u Val Gly Gly Val L u Ala Ala Leu Ala Ala
                    185             190             195
 5

    TAT TGC CTG TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC 630
    Tyr Cys Leu Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val
                    200             205             210

10  TTG TCC GGG AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC 675
    Leu Ser Gly Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr
                    215             220             225


    CAG GAG TTC GAT GAG ATG GAA GAG AAG GAG ACA GAG
15  Gln Glu Phe Asp Glu Met Glu Glu Lys Glu Thr Glu
                    230
```

(2) INFORMATION FOR SEQ ID NO:10:

20   (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 855 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

25

      (ii) MOLECULE TYPE: cDNA

      (ix) FEATURE:
        (A) NAME/KEY:

30

```
    ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC ACG GAT CCG GCG CCC
    Met Arg Gly Ser His His His His His His Thr Asp Pro Ala Pro
    1               5               10              15


35  ATC ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG TGT ATA    45
    Il  Thr Ala Tyr Ala Gln Gln Thr Arg Gly L u Leu Gly Cys Il
                    20              25              30
```

```
    ATC ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG GGT GAG    90
    Ile Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu Gly Glu
                35              40              45


 5  GTC CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA ACG TGC    135
    Val Gln Ile Val Ser Thr Ala Thr Gln Thr Phe Leu Ala Thr Cys
                50              55              60


10  ATC AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA ACG AGG    180
    Ile Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly Thr Arg
                65              70              75


15  ACC ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT ACC AAT    225
    Thr Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr Thr Asn
                80              85              90


    GTG GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT TCC CGC    270
20  Val Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly Ser Arg
                95              100             105


    TCA TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC CTG GTT    315
    Ser Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val
25              110             115             120


    ACG AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT GAT AGC   360
    Thr Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser
30              125             130             135


    AGG GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA AAA GGC   405
    Arg Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly
35              140             145             150
```

```
TCC TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC GTG GGC 450
Ser Ser Gly Gly Pr  Leu L u Cys Pro Ala Gly His Ala Val Gly
                    155             160             165


CTA TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG GCG GTG 495
Leu Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys Ala Val
                    170             175             180


GAC TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA TCC CCG 540
Asp Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg Ser Pro
                    185             190             195

GGG GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG TAT TGC 585
Gly Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala Tyr Cys
                    200             205             210


CTG TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC TTG TCC 630
Leu Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val Leu Ser
                    215             220             225


GGG AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC CAG GAG 675
Gly Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr Gln Glu
                    230             235             240


TTC GAT GAG ATG GAA GAG AAG GAG ACA GAG 705
Phe Asp Glu Met Glu Glu Lys Glu Thr Glu
                    245             250
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double

(D) TOPOLOGY: double

(ii) MOLECULE TYPE: cDNA

5
```
GA TCA   CCG GTC TAG ATCT
    T  GGC CAG ATC TAGA
```

(2) INFORMATION FOR SEQ ID NO:12:

10   (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 28 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: double
            (D) TOPOLOGY: linear

15
        (ii) MOLECULE TYPE: cDNA

        (ix) FEATURE:
            (A) NAME/KEY:

20
```
CCG GTC CGG AAG AAA AAG AGA CGC TAG C
    AG GCC TTC TTT TTC TCT GCG ATC G
```

(2) INFORMATION FOR SEQ ID NO:13:

25
        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 79 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: double
30   (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: cDNA

        (ix) FEATURE:
35   (A) NAME/KEY:
```
CCG GCA  ATT ATA CCT GAC AGG GAG GTT CTC TAC CAG GAA TTC
    GT  TAA TAT GGA CTG TCC CTC CAA GAG ATG GTC CTT AAG
```

```
GAT GAG ATG GAA GAG TGC CGG AAG AAA  AAG AGA CGC A
CTA CTC TAC CTT CTC ACG GCC TTC TTT  TTC TCT GCG TTC GA
```

(2) INFORMATION FOR SEQ ID NO:14:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 14 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: polypeptide

   (ix) FEATURE:
       (A) NAME/KEY: NS4A Active Mutant


```
Gly Cys Val Val Ile Val Gly Arg Ile Val Leu Ser Gly Lys
              5                   10
```

(2) INFORMATION FOR SEQ ID NO:15:

   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 13 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: polypeptide

   (ix) FEATURE:
       (A) NAME/KEY: NS4A Active Mutant


```
Cys Val Val Ile Val Gly Arg Ile Val Leu Ser Gly Lys
            5                   10
```

(2) INFORMATION FOR SEQ ID NO:16:

   (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

(ix) FEATURE:

(A) NAME/KEY: Soluble 5A/5B Substrate

```
Asp Thr Glu Asp Val Val Cys Cys Ser Met Ser Tyr Thr Trp Thr
                      5                 10                 15
Gly Lys
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

(ix) FEATURE:

(A) NAME/KEY: Mutant 5A/5B Substrate

```
Asp Thr Glu Asp Val Val Ala Cys Ser Met Ser Tyr Thr Trp Thr
                      5                 10                 15
Gly
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 amino acids

(B) TYPE: amino acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

5

    (ix) FEATURE:

        (A) NAME/KEY: Mutant Soluble 5A/5B Substrate

```
Asp Thr Glu Asp Val Val Ala Cys Ser Met Ser Tyr Thr Trp Thr
```
10   5   10   15

```
Gly Lys
```

2) INFORMATION FOR SEQ ID NO:19:

15   (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 18 amino acids

        (B) TYPE: amino acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

20

    (ii) MOLECULE TYPE: polypeptide

    (ix) FEATURE:

        (A) NAME/KEY: Soluble 5A/5B Substrate

25

```
Asp Thr Glu Asp Val Val Cys Cys Ser Met Ser Tyr Thr Trp Thr
```
                    5   10   15

```
Gly Lys Tyr
```
2) INFORMATION FOR SEQ ID NO:20:

30

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 18 amino acids

        (B) TYPE: amino acid

        (C) STRANDEDNESS: single

35   (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: polypeptide

(ix) FEATURE:

    (A) NAME/KEY: Soluble 5A/5B Substrate

```
5    Asp Thr Glu Asp Val Val Ala Cys Ser Met Ser Tyr Thr Trp Thr
                      5                 10                  15
     Gly Lys Tyr
```

2) INFORMATION FOR SEQ ID NO:21:

    (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 20 amino acids

       (B) TYPE: amino acid

       (C) STRANDEDNESS: single

       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: polypeptide

    (ix) FEATURE:

       (A) NAME/KEY: Soluble 4B/5A Substrate

```
     Trp Ile Ser Ser Glu Cys Thr Thr Pro Cys Ser Gly Ser Trp Leu
                      5                 10                  15
     Arg Asp Ile Trp Asp
```

2) INFORMATION FOR SEQ ID NO:22:

    (i) SEQUENCE CHARACTERISTICS:

       (A) LENGTH: 13 amino acids

       (B) TYPE: amino acid

       (C) STRANDEDNESS: single

       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: polypeptide

    (ix) FEATURE:

       (A) NAME/KEY: histidine tag

Met Arg Gly Ser His His His His His Thr Asp Pr
              5   10

2) INFORMATION FOR SEQ ID NO:23:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 9 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: polypeptide

    (ix) FEATURE:
      (A) NAME/KEY: hydrophilic tail

Arg Lys Lys Lys Arg Arg Lys Leu Asn
              5

2) INFORMATION FOR SEQ ID NO:24:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 4 amino acids
      (B) TYPE: amino acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: polypeptide

    (ix) FEATURE:
      (A) NAME/KEY: hydrophilic tail

Lys Glu Thr Glu

2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

    (A) LENGTH: 9 amino acids

    (B) TYPE: amino acid

5   (C) STRANDEDNESS: single

    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: polypeptide

10   (ix) FEATURE:

    (A) NAME/KEY: hydrophilic tail


```
Trp Ile Ser Ser Glu Cys Thr Thr Pro Cys Ser Gly Ser Trp Leu
```
15   5   10   15


```
Arg Asp Ile Trp Asp
```
        20

20   (2) INFORMATION FOR SEQ ID NO:26:

    (i) SEQUENCE CHARACTERISTICS:

      (A) LENGTH: 162 base pairs

      (B) TYPE: nucleic acid

25   (C) STRANDEDNESS: single

      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

30   (ix) FEATURE:

      (A) NAME/KEY: NS4A Mutant


```
GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG TAT TGC CTG    45
Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala Tyr Cys Leu
```
35 1   5   10   15


```
TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC TTG TCC GGG    90
Ser Thr Gly Cys Val Val Il  Val Gly Arg Ile Val Leu S r Gly
```

```
                    20                      25                      30

        AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC CAG GAG TTC   135
        Lys Pro Ala Il  Ile Pro Asp Arg Glu Val Leu Tyr Gln Glu Phe
 5                          35                      40                      45


        GAT GAG ATG GAA GAG TGC
        Asp Glu Met Glu Glu Cys
                            50
10
```

(2) INFORMATION FOR SEQ ID NO:27:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 810 base pairs
15   (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA
20

    (ix) FEATURE:
      (A) NAME/KEY: pNB182Δ4AHT

```
25      ATG AGA GGA TCG CAT CAC CAT CAC CAT CAC ACG GAT CCG CCC ATC   45
        Met Arg Gly Ser His His His His His His Thr Asp Pro Pro Ile
        1               5               10                      15


        ACG GCG TAC GCC CAG CAG ACG AGA GGC CTC CTA GGG TGT ATA ATC   90
30      Thr Ala Tyr Ala Gln Gln Thr Arg Gly Leu Leu Gly Cys Ile Ile
                            20                      25                      30


        ACC AGC CTG ACT GGC CGG GAC AAA AAC CAA GTG GAG GGT GAG GTC   135
        Thr Ser Leu Thr Gly Arg Asp Lys Asn Gln Val Glu Gly Glu Val
35                          35                      40                      45
```

```
        CAG ATC GTG TCA ACT GCT ACC CAA ACC TTC CTG GCA ACG TGC ATC   180
        Gln Ile Val Ser Thr Ala Thr Gln Thr Ph  Leu Ala Thr Cys Ile
                        50              55              60


5

        AAT GGG GTA TGC TGG ACT GTC TAC CAC GGG GCC GGA ACG AGG ACC   225
        Asn Gly Val Cys Trp Thr Val Tyr His Gly Ala Gly Thr Arg Thr
                        65              70              75


10      ATC GCA TCA CCC AAG GGT CCT GTC ATC CAG ATG TAT ACC AAT GTG   270
        Ile Ala Ser Pro Lys Gly Pro Val Ile Gln Met Tyr Thr Asn Val
                        80              85              90


15      GAC CAA GAC CTT GTG GGC TGG CCC GCT CCT CAA GGT TCC CGC TCA   315
        Asp Gln Asp Leu Val Gly Trp Pro Ala Pro Gln Gly Ser Arg Ser
                        95              100             105


20      TTG ACA CCC TGC ACC TGC GGC TCC TCG GAC CTT TAC CTG GTT ACG   360
        Leu Thr Pro Cys Thr Cys Gly Ser Ser Asp Leu Tyr Leu Val Thr
                        110             115             120


25      AGG CAC GCC GAC GTC ATT CCC GTG CGC CGG CGA GGT GAT AGC AGG   405
        Arg His Ala Asp Val Ile Pro Val Arg Arg Arg Gly Asp Ser Arg
                        125             130             135


30      GGT AGC CTG CTT TCG CCC CGG CCC ATT TCC TAC CTA AAA GGC TCC   450
        Gly Ser Leu Leu Ser Pro Arg Pro Ile Ser Tyr Leu Lys Gly Ser
                        140             145             150


35      TCG GGG GGT CCG CTG TTG TGC CCC GCG GGA CAC GCC GTG GGC CTA   495
        S r Gly Gly Pro L u Leu Cys Pr  Ala Gly His Ala Val Gly Leu
                        155             160             165
```

TTC AGG GCC GCG GTG TGC ACC CGT GGA GTG ACC AAG GCG GTG GAC 540
Phe Arg Ala Ala Val Cys Thr Arg Gly Val Thr Lys Ala Val Asp
                   170   175   180

TTT ATC CCT GTG GAG AAC CTA GAG ACA ACC ATG AGA TCC CCG GGG 585
Phe Ile Pro Val Glu Asn Leu Glu Thr Thr Met Arg Ser Pro Gly
                   185   190   195

GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG TAT TGC CTG 630
Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala Tyr Cys Leu
                   200   205   210

TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC TTG TCC GGG 720
Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val Leu Ser Gly
                   215   220   225

AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC CAG GAG TTC 765
Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr Gln Glu Phe
                   230   235   240

GAT GAG ATG GAA GAG TGC CGG AAG AAA AAG AGA CGC AAG CTT AAT 810
Asp Glu Met Glu Glu Cys Arg Lys Lys Lys Arg Arg Lys Leu Asn
                   245   250   255

(2) INFORMATION FOR SEQ ID NO:28:

    (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 162 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

    (A) NAME/KEY: Native NS4A

```
    TCA ACA TGG GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG 45
5   Ser Thr Trp Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala
    1               5                   10                  15


    TAT TGC CTG TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC 90
    Tyr Cys Leu Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val
10                  20                  25                  30


    TTG TCC GGG AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC 135
    Leu Ser Gly Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr
                    35                  40                  45

15
    CAG GAG TTC GAT GAG ATG GAA GAG TGC
    Gln Glu Phe Asp Glu Met Glu Glu Cys
                    50
```

20

(2) INFORMATION FOR SEQ ID NO:28:

    (i) SEQUENCE CHARACTERISTICS:

        (A) LENGTH: 162 base pairs

25   (B) TYPE: nucleic acid

        (C) STRANDEDNESS: single

        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

30

    (ix) FEATURE:

        (A) NAME/KEY: Native NS4A

```
    TCA ACA TGG GTG CTC GTT GGC GGC GTC CTG GCT GCT CTG GCC GCG 45
    Ser Thr Trp Val Leu Val Gly Gly Val Leu Ala Ala Leu Ala Ala
35  1               5                   10                  15
```

```
TAT TGC CTG TCA ACA GGC TGC GTG GTC ATA GTG GGC AGG ATT GTC 90
Tyr Cys Leu Ser Thr Gly Cys Val Val Ile Val Gly Arg Ile Val
             20                  25                  30
```

```
5   TTG TCC GGG AAG CCG GCA ATT ATA CCT GAC AGG GAG GTT CTC TAC 135
    Leu Ser Gly Lys Pro Ala Ile Ile Pro Asp Arg Glu Val Leu Tyr
                 35                  40                  45
```

```
    CAG GAG TTC GAT GAG ATG GAA GAG TGC
10  Gln Glu Phe Asp Glu Met Glu Glu Cys
                 50
```

(2) INFORMATION FOR SEQ ID NO:29:

15   (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 33 amino acid residues
           (B) TYPE: nucleic acid
           (C) STRANDEDNESS: single
           (D) TOPOLOGY: linear

20
        (ii) MOLECULE TYPE: polypeptide

        (ix) FEATURE:
           (A) NAME/KEY: Carboxl 33 mer of NS4A

25
```
Cys Val Val Ile Val Gly Arg Ile Val Leu Ser Gly Lys Pro Ala
                 5                  10                  15
```

```
Ile Ile Pro Asp Arg Glu Val Leu Tyr Gln Glu Phe Asp Glu Met
30               20                  25                  30
```

```
Glu Glu Cys
```

35 (2) INFORMATION FOR SEQ ID NO:30:

        (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 33 amino acid residues

(B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

5   (ii) MOLECULE TYPE: polypeptide

   (ix) FEATURE:
      (A) NAME/KEY: Carboxl 33 mer of NS4A of HCV-BK strain

10   Ser Val Val Ile Val Gly Arg Ile Ile Leu Ser Gly Arg Pro Ala
                      5   10   15

      Ile Val Pro Asp Arg Glu Leu Leu Tyr Gln Glu Phe Asp Glu Met
                     20   25   30

15
      Glu Glu Cys

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WE CLAIM:

5   1. A soluble HCV NS3 protease which is bacterially produced, denatured and refolded to produce soluble, active HCV NS3 protease.

2. A soluble HCV protease comprising HCV NS3 protease fused to a solubilizing motif.

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3. The soluble HCV protease of claim 2 wherein the solubilizing motif is a polypeptide comprising ionizable amino acids which, when fused to the HCV NS3 protease produces a protein which is soluble when expressed in *E. coli.*

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4. The soluble HCV protease of claim 3 wherein the solubilizing motif is comprised of Lys or Arg amino acid residues.

5. The soluble HCV protease of claim 2 wherein the solubilizing motif
20   is defined by SEQ ID NO: 2, SEQ ID NO: 23 or SEQ ID NO: 24.

6. The soluble HCV protease of claim 2 having a sequence defined by SEQ ID NO 3 or SEQ ID NO 4.

25   7. An HCV protease comprising HCV NS3 protease fused to an HCV NS4A cofactor wherein said cofactor has been modified by deletion or substitution of one or more amino acid residues to prevent cleavage by the HCV NS3 protease.

30   8. The HCV protease of claim 7 further comprising a solubilizing motif fused to said protease.

9. The soluble HCV protease of claim 8 wherein the solubilizing motif is a polypeptide comprising a sequence of ionizable amino acids which,
35   when fused to said HCV protease produces a protein that is soluble when expressed in *E. coli.*

10. The soluble HCV protease of claim 9 wherein the sequence of ionizable amino acids comprises Lys or Arg amino acid residues.

11. The soluble HCV protease of claim 9 wherein the sequence of ionizable amino acids is defined by SEQ ID NO: 2, SEQ ID NO: 23 or SEQ ID NO: 24.

12. The HCV protease of claim 8 having a sequence defined by SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10 or SEQ ID NO: 27.

13. A modified NS4A cofactor having a sequence defined by SEQ ID NO 14, SEQ ID NO 15 or SEQ ID NO 26.

14. An isolated nucleic acid or a vector encoding a polypeptide or protein of claims 1-13.

15. A host cell transformed or transfected with an isolated nucleic acid or vector of claim 14.

16. The host cell of claim 15 wherein the host cell is bacterial.

17 A method for making a soluble HCV protease comprising culturing the host cell of claims 15 or 16 under conditions in which the proteae is expressed.

18. An HCV NS3 protease having a polyhistidine tag attached to said protease, wherein the histidine tag is comprised of two or more histidine residues.